EXHIBIT V

in 20 neurones. Thus, 30% of the inward current was carried by Na^+. This Na^+ current was isolated after blocking Ca^{2+} current with verapamil (Knoll) in doses of $1–5\times10^{-5}$ M or by substituting Co^{2+} for Ca^{2+} (Fig. 2*d*). The Na^+ current has a transient peak and then inactivates although the time course may vary among different neurones.

After TTX or $Tris^+$ substitution for Na^+, the inward current was carried by Ca^{2+} (Fig. 2*c*). A component of the Ca^{2+} current elicited by small depolarisations showed little or no inactivation as previously reported[10]. At larger voltages the inward Ca^{2+} current also had a transient peak. The calcium was increased or decreased when extracellular calcium was doubled or halved, and reduced or abolished by when Co^{2+} was substituted for Ca^{2+} or verapamil was added. In TTX, the Ca^{2+} current was unchanged when $Tris^+$ was substituted for Na^+. Substitution of methanesulphonate ion for Cl^- had no effect on any of these currents.

Having established the presence of a TTX-sensitive Na^+ current, we tested the effects of trypsin (type III, Sigma) on this current in 20 neurones. The outward K^+ and inward Ca^{2+} currents were blocked and the inward Na^+ currents elicited by 20-mV voltage steps from −60 to +160 mV were recorded. Such Na^+ currents could be blocked by TTX or substitution of $Tris^+$ for Na^+ (Fig. 3*a*). Neurones with the inward Na^+ current separated in this way were then exposed to 0.1% trypsin for about 2–4 min. Exposure to these doses of trypsin for this period increased membrane resistance silghtly but did not affect the magnitude of the inward currents or their reversal potentials. In some instances, the time course was slightly altered. TTX now had no effect on the Na^+ current (Fig. 3*d*). But substitution of $Tris^+$ for Na^+ still blocked the inward current (Fig. 3*e*).

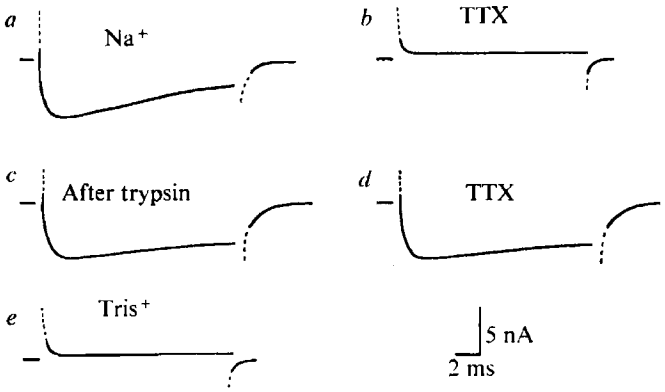

Fig. 3 Na^+ current in a *Helix* neurone, its blockage by TTX and the effects of trypsin on the Na current and the TTX blockage. $V_H = -60$ mV. All currents elicited by voltage steps to +25 mV. Internal perfusion with Cs aspartate, external solution contains CsCl (see text). Ca^{2+} currents blocked by verapamil. *a*, Control Na^+ current. *b*, After 5 min in 3×10^{-5} M TTX. Only the outward leakage current persists. *c*, After recovery in TTX-free solution for 30 min and exposure to 0.1% trypsin for 3 min. The magnitude of the inward current is unchanged but inactivates more slowly. This is not, however, a consistent finding. *d*, After 10 min exposure to 1.5×10^{-4} M TTX in trypsin-free solution. TTX no longer blocks Na^+ current. *e*, Substituion of $Tris^+$ for Na^+ in the external solution eliminates the Na^+ current leaving a small outward leakage current.

To examine whether trypsin acted on the Ca^{2+} current, we separated it from the Na^+ and K^+ currents and compared the inward currents before and after enzyme treatment. Equivalent exposures which abolished TTX-sensitivity had no effect on either calcium currents or their blockage by verapamil or Co^{2+}. Exposures of 10 min, however, reduced membrane resistance and Ca^{2+} currents as well as the TTX-insensitive Na^+ current.

Trypsin destroys the TTX-sensitivity of the neuronal membrane without affecting the magnitude and time course of the inward currents. This accounts for the absence of TTX sensitivity in snail neurones[13] pretreated with this enzyme. The action is specific since Na^+ current persists and the Ca^{2+} current is unaffected. The external application of trypsin to lobster giant axon[1], or α-chymotrypsin to squid giant axon[4], was also without effect on the membrane's electrical properties although another enzyme, Pronase, when applied internally to squid giant axon, removes Na^+ inactivation selectively[3]. If the guanidinium group of the TTX molecule blocks the Na^+ channel at the selectivity filter[20], the persistence of a TTX-insensitive inward Na^+ current with an unchanged reversal potential suggests that the selectivity filter has not been affected by trypsin. Thus, trypsin may act on the part of the channel occupied by the remainder of the TTX molecule and this portion probably contains lysine and/or arginine residues. It is also interesting that modifications of TTX at carbon positions 4 and 9, which are close to the guanidinium group, greatly reduce the potency of the toxin[21]. The lack of TTX sensitivity of some excitable cells, such as puffer fish neurones[22] and denervated skeletal muscle[23] cells, may have a similar basis.

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Formation *in vivo* of volatile *N*-nitrosamines in man after ingestion of cooked bacon and spinach

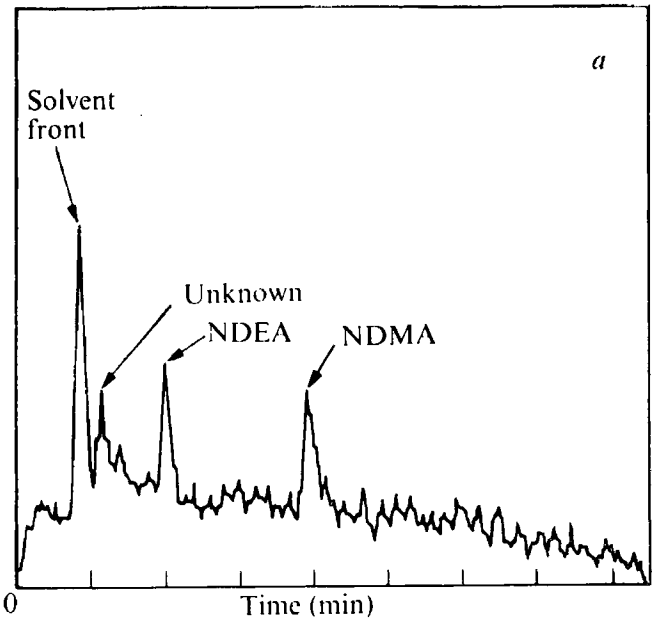
N-NITROSAMINES, which are thought to be causally related to human cancer[1], have been found in $\mu g\,kg^{-1}$ concentrations in polluted air[2,3], in water[4] and in tobacco products[5]. Foodstuffs such as cooked bacon, preserved with nitrite, have long been known to contain $\mu g\,kg^{-1}$ amounts of volatile *N*-nitrosamines formed during cooking[6]. *In vivo* formation of *N*-nitrosamines, after ingestion of suitable amine precursors and nitrite, has been demonstrated in

laboratory animals[7]. *In vivo* formation in human subjects with hypoacidity, gavaged with relatively massive amounts of diphenylamine plus nitrite, has been demonstrated through detection of *N*-nitrosodiphenylamine in the stomach contents[8]. *In vivo* nitrosation after ingestion of conventional foods has not been demonstrated so far, either in animals or man. We now report *in vivo* formation of volatile *N*-nitrosamines in man after ingestion of a midday meal consisting of a bacon, spinach and tomato sandwich and beer.

Blood (20 ml) was taken on the day before the test. On the day of the test, 20 ml of blood was taken 50 min before the noon meal. Lunch consisted of 310 g of spinach, 170 g of cooked bacon, 200 g of tomatoes, 120 g of bread and 460 g of beer. One per cent of the total lunch was analysed for volatile *N*-nitrosamines. Blood samples (20 ml) were taken 35, 65, 162 and 220 min after the meal. A final control sample was taken at 1,300 min. Samples were poured immediately into liquid nitrogen. Less than 20 s elapsed between the end of collection and freezing in liquid nitrogen. Mineral oil was added to the whole blood, which was analysed for volatile *N*-nitrosamines by standard procedures[9]. Control experiments were performed by recovering *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosopyrrolidine (NPYRR) at the 0.4-$\mu g\ kg^{-1}$ level (0.4 p.p.b.) from whole human blood (obtained from a blood bank). The blood from the bank contained no voltaile *N*-nitrosamines.

The meal contained preformed NDMA and NPYRR. The total amount of preformed NDMA ingested was 1,600 ng. Identification of NDMA and NDEA was confirmed using both TEA-GLC and TEA-HPLC. Figure 1 shows the chromatographic traces of the blood sample taken 162 min after the meal. The TEA-GLC trace shows only two peaks, those at the retention time of NDMA and NDEA. The TEA-HPLC trace shows three peaks, two eluting at the same retention time as NDMA and NDEA (note that the peak order is reversed). Furthermore, the quantities of NDMA and NDEA are identical for both the GLC and HPLC systems. As a further confirmatory step, the NDMA and NDEA peaks eluting on the HPLC were

Fig. 1 *a*, High-pressure-liquid-chromatogram for a 10-µl extract of whole human blood. The TEA-HPLC was constructed[10] by combining an injector (Waters Associates, model UK6), with a high-pressure pump (Waters Associates, model 6000A) and a thermal energy analyser (Thermo Electron, model TEA-HPLC 502). A µm Porasil column was used with a 5% acetone–95% 2,3,4-trimethylpentane solvent system at a flow rate of 2 ml min^{-1}. TEA attentuation was × 2. *b*, Gas–liquid chromatogram for a 10-µl extract of whole human blood. A single-column isothermal gas chromatograph was constructed[11] from 14 feet of stainless steel tubing (outer diameter 1/8 inch), packed with 10% Carbowax 20 M on Chromasorb W, 80–100 mesh. The column temperature was 180 °C, with Ar carrier gas at a flow of 25 ml min^{-1}. A thermal energy analyser (Thermo Electron, model TEA–502) was used as the detector. TEA attenuation was × 2.



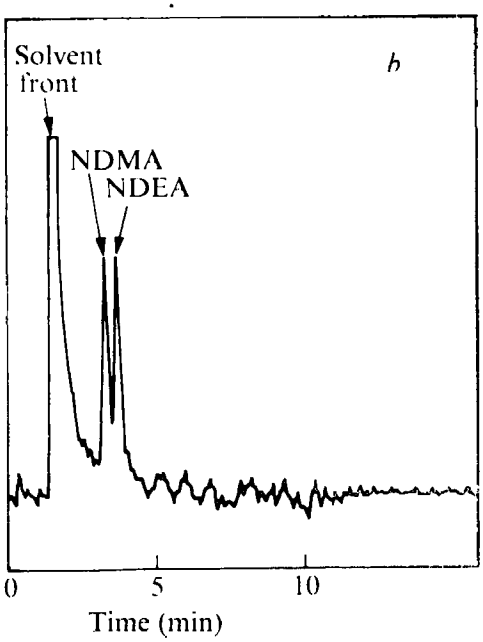
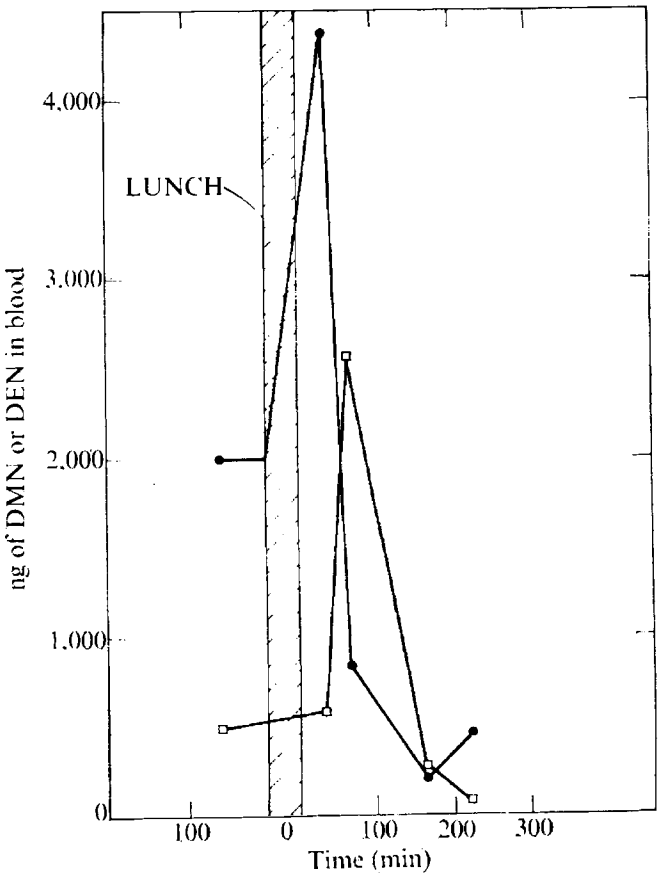


Fig. 2 *In vivo* nitrosation in man after ingestion of bacon, spinach, tomato and beer. ●, NDMA; □, NDEA.



isolated and reconcentrated and then injected on to the GLC. The peak corresponding to NDMA on the HPLC gave only a single peak on the GLC, corresponding to NDMA. The same was true for NDEA. Given the selectivity of the TEA, evidence for the identity of volatile *N*-nitrosamines based on parallel TEA-GLC and TEA-HPLC procedures can be taken as confirmatory[10,11].

The amount of NDMA and NDEA present as a function of time in the blood (assuming total body blood is 5,640 ml) is shown in Fig. 2. Before the meal the blood contained 2,000 ng of NDMA and 510 ng of NDEA; 35 min after the meal NDMA had increased to 4,350 ng and NDEA to 570 ng, and 65 min after the meal, NDMA had fallen to 860 ng while NDEA had increased to 2,600 ng. At 162 min, both had decreased below the levels observed before the meal. At 1,300 min there was 760 ng of NDMA in the blood; NDEA was not present. NPYRR was not detected in the blood either before or after the meal.

These amounts of NDMA in human blood imply that the compound was being formed *in vivo* in quantities greater than those present before the meal. The total amount of NDMA in the body may be considerably in excess of that reported here because we assumed that all NDMA was taken up in the blood alone—an appreciable amount may have been exhaled or absorbed in various tissues. NDEA was not found in the food, but it was formed *in vivo* after the meal. It is interesting that the content of NDEA was maximal after the concentration of NDMA had reached its maximum. Our experiments were carried out three times, giving similar results for NDMA, although the actual measurements differed in each case. NDEA was detected in only one of the three experiments. The source of the background NDMA in the blood is unknown; no background was observed if the volunteer had eaten a bland low nitrate–nitrite, high ascorbate diet for the previous 24 h.

The experiments reported here, although simple, demonstrate conclusively that *N*-nitrosamines can be formed *in vivo* in man after ingestion of conventional foodstuffs.

The ability routinely to detect nitrosation *in vivo* should make possible realistic tests of putative environmental carcinogens.

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Ferritinaemia in cancer

SMALL amounts of ferritin are found in serum in normal and pathological states[1,2], usually at levels directly related to the amount of tissue storage iron[3,4]. This relationship, however, does not hold in many cancers where grossly elevated levels can occur without a corresponding increase in iron stores[5–9]. These latter findings suggest a possible use of ferritinaemia in cancer diagnosis, but the value of this test has not been clearly established. Such use is complicated by the multiple potential sources of the serum ferritin which could affect its type as well as its amount. Much of the ferritin in cancer sera may reflect increased iron stores arising from chronic anaemia or from transfusions in treated patients. Some may also come from nonspecific tissue damage. The high levels of ferritin synthesis in some cancer cells[10] and the presence in serum of isoferritins characteristic of some cancer and foetal tissues[11,12], however, suggest that much of the ferritin may come directly from cancer cells. If so, selective quantitation of these isoferritins may provide specificity in possible serodiagnosis. The multiple isoferritins found in most human tissues seem to be hybrid molecules composed of different proportions of two subunit types[13]. One type predominates in liver and spleen isoferritins, and the other in the more acidic isoferritins in heart, tumours and HeLa cells[14]. Different isoferritin populations can be distinguished immunologically, apparently on the basis of their subunit composition[14,15]. Ferritin is usually assayed by immunological methods, with crystalline liver or spleen ferritins as immunising antigens and reference standards. These ferritins consist almost entirely of the liver-type subunit[15]. Consequently, most assays based on these ferritins have only a low reactivity for the more acidic 'carcinofoetal' isoferritins which may contain less than 20% of the liver-type subunit[14,15]. We have, therefore, tested a variety of cancer sera and compared the apparent levels of ferritin given by a standard 'liver-type' assay with levels given by an assay whose specificity is directed against the more acidic isoferritins[15]. We report here preliminary evidence that the latter type of assay may significantly improve the usefulness of ferritinaemia as a tumour marker.

Serum samples from untreated patients with various histologically confirmed malignancies were provided by Dr Elliot Alpert, Massachusetts General Hospital, Boston, Mass. The leukaemic sera were obtained from New England Medical Center, Boston. The samples were not graded according to the stage of disease or other pathological disorders. The levels of ferritin in these sera were estimated by two radioimmunoassays (RIA). One RIA reacts preferentially with liver-type isoferritins, the other with the more acidic isoferritins characteristic of heart and HeLa cells[15]. Antibodies for the 'liver-type' RIA were raised in rabbits against crystalline human liver ferritin consisting of approximately 90% of the liver-type subunit. Antibodies for the 'HeLa-type' RIA were developed against HeLa ferritin (>80% heart-type subunit). Before use, the anti-HeLa antiserum was absorbed with a natural apoferritin preparation (>95% liver-type subunit) to restrict its specificity to sites determined by the heart-type subunit[15].

The apparent level of the serum ferritin from 8 out of 12 different cancers was substantially higher when estimated with the 'HeLa-type' than with the 'liver-type' assay (Table 1). In most cases, the ratio of the values given by the two assays (HeLa–liver) was between 2 and 5. These ratios are markedly different from those obtained in non-malignant conditions, such as iron overload, where the ratio was less than 0.1. This differential indicates that the ferritin in these cancer sera contained substantially higher proportions of the heart-type subunit than the serum ferritins from iron overload. This conclusion must also apply even in cases where the apparent level given by the 'HeLa-type' RIA was slightly lower than that given by the 'liver-type' RIA (for example, cases 4, 21).